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13. ABSTRACT ***(Maximum 200 Words)***

Purpose: To test the working hypothesis that SHP-1 is essential for controlling growth and differentiation of mammary epithelial cells and that dysregulation of SHP-1 contributes to the development of breast cancer.

Scope: To biochemically and functionally characterize SHP-1 in human breast cancer cell lines and to define the biological function of SHP-1 in normal epithelial cells.

Major Findings: SHP-1 associates with the EGFR in an EGF stimulation-dependent manner. To further characterize the function of SHP-1, we have generated retroviruses encoding wild type and mutant forms of SHP-1 together with GFP. Infection of cell lines with these viruses will enable us to target a high percentage of the cell population and to identify infected cells. We have also generated a transgenic mouse encoding SHP-1 under the control of its hematopoietic promoter. Crossing this mouse in the *motheaten* background should allow us to study SHP-1-deficient epithelial cells in an otherwise "normal" mouse.

Significance: From our data, we are starting to understand SHP-1's role in epithelial cells. Moreover by using the reagents we generated, we expect to deepen our knowledge of SHP-1's role in epithelial cells and to learn how a dysregulated SHP-1 is potentially involved in the onset/progression of breast cancer.

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Ulrike Lorent 10/29/99

PI - Signature Date

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INTRODUCTION

While a strong correlation between the development of breast cancer and expression of certain protein tyrosine kinases (PTKs), such as members of the ErbB family of receptor kinases or the cytoplasmic c-Src kinase, has been observed, little is known about the role of protein tyrosine phosphatases (PTPs) in breast cancer. However, it is logical to speculate, that PTPs would balance the PTK activities and thereby counteract their tumor-promoting actions or unbalanced PTPs could be tumor-promoting themselves. SHP-1 is a cytoplasmic tyrosine phosphatase expressed exclusively in epithelial cells and the hematopoietic lineage. We chose to study SHP-1 as a possible mediator in the onset/progression of breast cancer for the following reasons: (1) In the hematopoietic system, the role of SHP-1 as a negative regulator has been well established. It is conceivable that SHP-1 has a similar role in epithelial cells, and its dysregulation could contribute to neoplasms arising in breast epithelial cells. Biochemical and functional characterization of SHP-1 in normal and transformed epithelial cells will be addressed as part of Tasks 1, 2 and 3. (2) In our preliminary studies, mice which lack one of the wild type SHP-1 alleles have a high incidence of breast tumors, suggesting a role for this phosphatase in the onset/progression of breast cancer. This hypothesis will be addressed in Tasks 3 and 4. The goal of this proposal is to rigorously examine the involvement of SHP-1 in the development of breast cancer in mice as a model system and in human primary breast tumors and cell lines.

BODY

Task 1 (Characterization of SHP-1 in human breast cancer lines and primary tumors)

To further biochemically characterize SHP-1 in epithelial cells and in particular in human breast tumor cell lines, we chose the cell line MDA-MB 468. In our preliminary analysis, we had shown that this cell line expresses SHP-1. Moreover, this cell line expresses the Epidermal

Growth Factor Receptor (EGFR), but no detectable levels of other members of the ErbB family (1). Thereby, it provides a system where a potential interaction between SHP-1 and the EGFR can be studied without the complication of generating heterodimers between the EGFR and other members of its family.

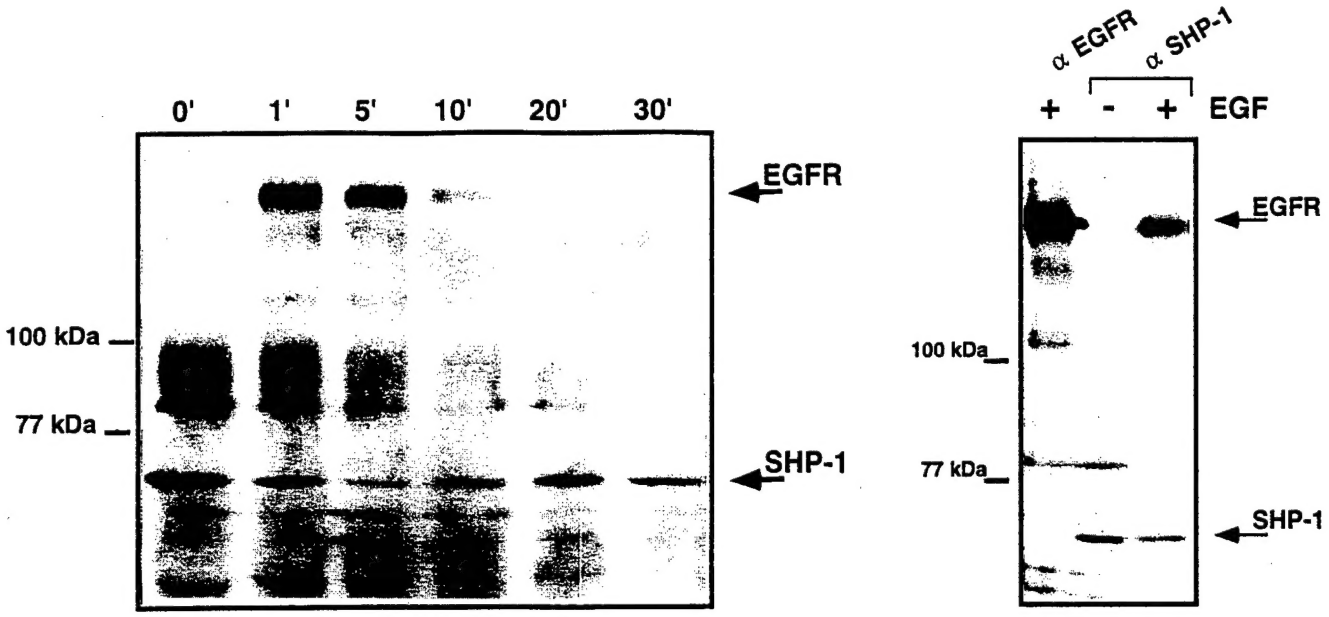

Figure 1: Association of SHP-1 with the EGFR upon EGF stimulation in the MDA-MB 468 cell line. (**A**) MDA-MB 468 cells were serum-starved (20 hrs.) before stimulation with 100 ng/ml EGF for the indicated times. SHP-1 was immuno-precipitated from 2 mg cell lysate using affinity-purified rabbit polyclonal anti-SHP-1 antibodies. The immunoprecipitated proteins were separated by 8% SDS-PAGE, and analyzed for their phosphotyrosine content by anti-phosphotyrosine immunoblotting. (**B**) MDA-MB 468 cells were serum-starved (20 hrs.) and stimulated with 100 ng/ml EGF for the 5 min.. EGFR or SHP-1 (as indicated) were immunoprecipitated from 2 mg cell lysates using the respective antibodies (anti-EGFR was purchased from Sigma). The immunoprecipitated proteins were separated by 8% SDS-PAGE, and analyzed for phosphotyrosine content by anti-phosphotyrosine immunoblotting.

As shown in Figure 1, we observed that upon EGF stimulation, SHP-1 associates with several tyrosyl phosphorylated proteins (Fig. 1A) one of which co-migrates with the EGFR

(Fig. 1B) suggesting an association between SHP-1 and the EGFR. It is interesting to note that the time course of overall EGFR tyrosyl phosphorylation differs from the time course of SHP-1 associated EGFR. While the majority of phosphorylated EGFR associates with SHP-1 immediately following stimulation with EGF (1 min and 5 min.) and decreases after 5 min., the tyrosyl phosphorylation of the total EGFR peaks between 5 and 10 min, and is sustained up to 20 -30 min. following stimulation (data not shown). This difference emphasizes that the observed association between SHP-1 and the EGFR is specific and does not reflect a general association of SHP-1 with tyrosyl phosphorylated proteins. Presently, we are trying to address what the differences between SHP-1-bound and unbound phosphorylated EGFR are.

Taken together, we have shown that SHP-1 interacts with the EGFR in an EGF stimulation-dependent way. To our knowledge, this is the first time that such an interaction has been shown in a more physiological setting where none of the binding partners was overexpressed. This regulated interaction supports our hypothesis that SHP-1 might be a regulator of EGF-induced signaling and might thereby also affect the onset/progression of breast tumor development.

Task 2 (Defining the function of SHP-1 in human breast cancer cells)

We had proposed to functionally characterize SHP-1 in epithelial cells by introducing wild type as well as a panel of mutants of SHP-1 into human breast cancer cell lines. The goal of this Technical Objective was to identify SHP-1's "normal" function by interfering with it. Based on preliminary studies, we concluded that infection of the cell lines with a retrovirus would provide us with a better system than "regular" transfection methods. We chose a mouse stem cell virus (MSCV) containing an IRES sequence followed by the gene for the Green Fluorescence Protein (GFP). Expression of GFP will allow us to easily assess the percentage of infected cells.

Moreover, we will be able to identify on a single cell basis infected cells using flow cytometric analysis. To generate infectious retroviruses of this packaging-deficient virus, the retroviral plasmid has to be co-transfected with plasmids encoding the envelope, gag, and polymerase genes. We chose to use the envelope gene of the Vasicular Stomatitis Virus (VSV) to generate a highly potent amphitropic virus. In our initial experiments using the epithelial cell line HeLa as target cells for this retrovirus, we observed a 60-90% infection rate (Figure 2). Such high percentages of infected cells will enable us to do the functional studies proposed in our original application. At the moment, we are generating a panel of retroviral vectors containing wild type and mutant forms of SHP-1.

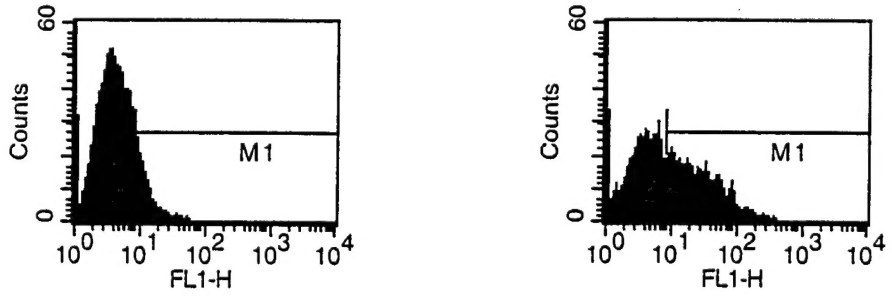

Figure 2: Flow cytometric analysis of HeLa cells uninfected (left panel) or infected (right panel) with VSV packaged MSCV virus. The GFP-expressing population (representing infected cells) is marked as M1. These data represent an example of a single round of infection. Several consecutive rounds of infection can increase the infection rate to up to >90%.

Task 3 (Defining the biological function of SHP-1 in normal epithelial cells)

We had proposed to generate a transgenic mouse expressing SHP-1 under the control of its hematopoietic promoter. We obtained two female founder mice carrying the transgene. However, only one of the mice delivered off-spring carrying the transgene. Mice carrying the

transgene are viable and, at least based on what we have observed so far, seem normal. By further breeding, we generated a stock of these transgenic mice and, as originally proposed, we are now crossing them into the *motheaten* background. We expect to thereby generate a partially rescued *motheaten* mouse. This mouse should allow us to study SHP-1-deficient epithelial cells in an otherwise "normal" background. Moreover, it will be interesting to see whether these mice will develop breast tumors at an earlier age.

Task 4 (Analysis of breast tumors in *me*/+ mice)

In our preliminary studies, we had observed that retired *me*/+ female mice display an unusual high frequency of breast tumors. As a control, we observed more closely +/+ mice of the same C3HeB/FeJLe-*a/a* strain. At this point, we have not observed a similar high frequency of breast tumors in these +/+ mice, whereas we continue to observe breast tumor formation in the *me*/+ mice. Even though we were assured by Jackson Laboratories, Maine, that these mice are MMTV-free, we will confirm this by flow cytometric analysis of the their T cell repertoire. Production of MMTV leads to expression of superantigens which in turn cause the deletion of certain T cell receptor Vβ chains. In the case of MMTV-C3H, these are Vβ chains 14 and 15 (2, 3). Therefore, presence of these Vβ chains would confirm the absence of MMTV.

Key Research Accomplishments

- SHP-1 associates with the tyrosyl phosphorylated EGFR in an EGF stimulation-dependent manner. (Task 1)
- Generation of retroviruses which encode wild type and mutant forms of SHP-1 together with GFP allowing identification of infected cells. 60-90 % of epithelial cell population can be infected. (Task 2)
- Generation of transgenic founder mouse carrying cDNA for SHP-1 under the control of its hematopoietic promoter. (Task 3)
- Increased frequency of breast tumors in C3HeB/FeJLe-*a/a* female *me/+* mice compared to +/+ mice is observed. (Task 4)

Reportable Outcomes ---- not yet finished

Conclusions

The data obtained during the last year as part of this study show that SHP-1 participates in the signal transduction pathway downstream of the EGFR. Since members of the ErbB family of receptor kinases have been shown to play a role in the development of breast tumors, our findings suggest that SHP-1 might also play a role in this process. The reagents generated, such as the transgenic founder mouse and the SHP-1 carrying retroviruses should provide us with the needed tools to rigorously test our proposed working hypothesis that SHP-1 is involved in the development of breast cancer.

"So what": In our original grant application, we had proposed as a working hypothesis that SHP-1 is essential for controlling growth and differentiation of mammary epithelial cells and that dysregulation of SHP-1 contributes to the development of breast cancer. From the data we obtained so far, we are starting to gain an understanding of SHP-1's role in epithelial cells.

Moreover by using the reagents we generated and continuing our studies over the next years, we expect not only to deepen our knowledge of SHP-1's role in epithelial cells but also to learn how a dysregulated SHP-1 is potentially involved in the onset/progression of breast cancer. Moreover, the knowledge of SHP-1's dysfunction and its consequences in certain breast tumors might allow us to use it as a diagnostic and/or a prognostic marker. This might also have implications for possible future therapies.

References

1. J. S. Biscardi, B. A.P., S. J. Parsons, *Molecular Carcinogenesis* **21**, 261 (1998).
2. P. Marrack, E. Kushnir, K. J., *Nature* **1991**, 524 (1991).
3. Y. Choi, J. W. Kappler, P. Marrack, *Nature* **350**, 203 (1991).